Koisio technology-produced water significantly decreases H_2O_2-produced oxidative stress in both cell culture studies and animal model studies

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Abstract

Oxidative stress plays critical pathological roles in a number of diseases. Our previous study showed that the water that was produced by a preliminary Koisio technology showed certain levels of antioxidant capacity. Since the preliminary Koisio technology was not a standardized procedure, it is necessary to determine if the water that is produced through standardized procedures of Koisio technology has the antioxidation capacity. In this study we determined if the water produced by standardized Koisio technology is capable of decreasing cellular oxidative stress. Our study obtained the following findings: First, after treatment of microglial BV2 cells with H_2O_2, the cells cultured in the culture media produced by standardized Koisio technology-produced water had decreased level of oxidative stress, compared to the cells cultured in the culture media produced by regular water; second, after treatment of the cells with H_2O_2, there was lower levels of apoptosis of the cells cultured in the culture media made by standardized Koisio technology-produced water, compared to the cells in the culture media made by regular water; and the third, drinking of the water produced by standardized Koisio technology led to significantly decreased activity of myeloperoxidase (MPO) in the mouse model of Dextran Sulfate Sodium salt (DSS)-induced acute colitis, which is a key oxidative stress-generating enzyme in the inflammatory processes. Collectively, both our *in vitro* studies and *in vivo* studies have indicated that the water produced by standardized Koisio technology has significant antioxidant capacity.

Keywords: Oxidative stress; Koisio water; H_2O_2; cell death; mouse model

Introduction

Oxidative stress is a crucial pathological factor in multiple major diseases including stroke, myocardial ischemia and diabetes [1-3]. Oxidative stress is also an important factor in aging [4]. Oxidative stress can produce numerous cellular injuries and cell death by impairing cell membrane, proteins and DNA [5]. Therefore, it is of great significance to search for novel methods or agents that have antioxidant capacity.

Water is a major component of life, which occupies approximately 70 % of the space of human body. The quality of drinking water can significantly impact human health [6, 7]. Due to the significance of drinking water, it is crucial to invent novel strategies to modulate the properties of water, so as to discover novel biological functions of water.

Our previous study showed that the water that was produced by a preliminary Koisio technology showed certain levels of antioxidant capacity [8]. This study has proven the principle that Koisio technology-produced water has antioxidation capacity, which is a pivotal, fundamental finding regarding the functions of Koisio technology-produced water. However, since the preliminary Koisio technology was not a standardized procedure, it is necessary to conduct studies to determine if the water that is produced through standardized procedures of Koisio technology has the antioxidation capacity, so as to provide solid quantifications of the antioxidation capacity of the water. Since standardized procedures of Koisio technology for producing water have been established, in this study we determined the capacity of the water produced by

standardized Koisio technology to decrease oxidative stress in both cell culture studies and animal studies.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted. Koisio water (KW) was produced by the technical experts of Shanghai Koisio Food Industry Co. (Shanghai, China) according to standard procedures.

Methods

Cell culture models

The murine microglia cell line BV2 cells were purchased from the Chinese Academy of Sciences Cell Bank. BV2 cells were grown in KW-based Dulbecco's modified Eagle medium (DMEM) or ddH_2O-based DMEM, both of which were supplemented with 10% heat-inactivated fetal bovine serum (FBS, A5669701, Gibco), 100U/mL penicillin, and 0.1 mg/mL streptomycin (15140122, Gibco) at 37 °C in humidified 5% CO_2 atmosphere.

Production of cell culture media

The culture media were prepared according to the manufacturer's instructions. In brief, 13.5 g of DMEM powder (12800017, Gibco) was added to 950 mL sterilized

purified water or KW, subsequently 1 N HCl was used to adjust the pH to around 4.1 - 4.2, followed by transferring 47.5 mL of media to a 50 mL centrifuge tube. Approximately 1.5 mL of 7.5 % $NaHCO_3$ solution was added to each 50 mL tube to adjust the pH to 7.2 – 7.4. Finally, sterile syringe filters with a 0.22 μm pore size (BE-PES-22, Biosharp) were used to filter the culture media.

Determinations of intracellular ROS levels

For Dichlorofluorescin (DCF) assay, 2,7-Dichlorofluorescin diacetate (DCFH-DA, Beyotime, China), a reactive oxygen species-specific fluorescent probe, was used to measure total intracellular ROS levels. After BV2 cells were cultured in either regular cell culture media or KW cell culture media for 24 h, the cells were exposed to the 1 mM H_2O_2 prepared in Minimum Essential Medium (MEM, 11095080, Gibco) for 1 h. After the treatment, the cells were cultured in regular cell culture media or KW cell culture media for 18 – 24 h. The cells were incubated with 20 μM DCFH-DA dissolved in DMEM without FBS for 30 min at 37°C in an incubator. After 3 times washes with phosphate buffered saline (PBS), the cells were analyzed by flow cytometry (FACS Aria; Becton Dickinson, Heidelberg, Germany) to detect the mean fluorescence intensity (MFI) with an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Flow cytometry-based annexin V/7-AAD assay

The flow cytometry assay was performed to determine the levels of early-stage

apoptosis, late-stage apoptosis, and necrosis using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. After BV2 cells were cultured in either regular cell culture media or KW cell culture media for 24 h, the cells were exposed to the 1 mM H_2O_2 prepared in MEM for 1 h. After the treatment, the cells were cultured in regular cell culture media or KW-based cell culture media for 18 – 24 h. Finally, BV2 cells were harvested by 0.25% trypsin-EDTA, washed by cold PBS twice and resuspended in binding buffer. 5 μL of Annexin V and 5 μL of 7-AAD were added into 100 μL of the cell suspensions. After incubation at room temperature for 20 min, the number of stained cells was assessed immediately by a flow cytometer (FACSAria II, BD Biosciences).

Animal model of acute colon inflammation

Male C57BL/6Slac mice at the weight between 18-24 g were administered with Koisio technology-produced water or regular water for 10 days. The mice were then administered with 3% (w:v) Dextran Sulfate Sodium salt (DSS) in Koisio technology-produced water or dH_2O for 7 days. On the final day, the colons were obtained for myeloperoxidase assay (MPO).

MPO Assay

MPO assay was conducted as described previously [9, 10]. The colon was weighted and placed separately in tube containing 0.5% hexadecyltrimethylammonium bromide in a 50 mM potassium phosphate buffer (4.35 g of dibasic potassium phosphate

and 3.4 g of monobasic potassium phosphate in 1 L of dH_2O, pH=6.0). The ratio of colon to buffer was 50 mg/ml. The colons were homogenized and centrifuged at 14,000 × g, 4 °C for 5 min. The supernatant was collected for test. Before measuring the plate, 10 µl supernatant of each sample was combined with 200 µl of an o-dianisidine solution (0.167 mg/mL *o*-dianisidine dihydrochloride, 0.0006% hydrogen peroxide in 5mM potassium phosphate buffer pH=6.0). Changes in absorbance were measured at 460nm and recorded at 30 s intervals for 5 min.

Statistical Analysis

Data were presented as mean ± SEM and analyzed by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test except where noted. *P* values less than 0.05 were considered statistically significant.

Results

1.KW showed significant capacity of decreasing ROS levels in the H_2O_2-treated BV2 microglial cells.

After BV2 cells were cultured in either regular cell culture media or KW-based cell culture media for 24 h, the cells were treated with 1 mM H_2O_2 for 1 h. After the treatment, the cells were cultured in regular cell culture media or KW-based cell culture media for 18 – 24 h. Subsequently FACS-based assay using DCFH as ROS probe was conducted. The FACS-based assay showed that the intracellular ROS level in the cells cultured in KW-based cell culture media was significantly lower than that in the cells

cultured in regular cell culture media (**Figs.1A and 1B**).

2. KW showed significantly greater capacity to decrease the apoptosis and necrosis in the H_2O_2-treated BV2 microglial cells.

After BV2 cells were cultured in either regular cell culture media or KW-based cell culture media for 24 h, the cells were exposed to the 1 mM H_2O_2 prepared in MEM for 1 h. After the treatment, the cells were cultured in regular cell culture media or KW-based cell culture media for 18 – 24 h. Subsequently FACS-based assay was conducted with Annexin V Apoptosis Detection Kit with 7-AAD. The FACS-based assay showed that the quantities of both early apoptotic cells and late-stage apoptotic cells cultured in KW-based cell culture media were significantly less than the cells cultured in regular cell culture media (**Figs. 2A and 2B**).

3. Drinking of KW led to significantly attenuated increases in MPO activity in the mouse model of DSS-induced acute colitis

In the mouse model of DSS-induced acute colitis which had drunken regular water, DSS induced significant increases in MPO activity, which was significantly attenuated in the mice that had drunk KW (**Fig.3**).

Discussion

Our study obtained the following findings: First, after treatment of microglial BV2 cells with H_2O_2, the cells cultured in the KW-based culture media had decreased

level of oxidative stress, compared to the cells cultured in the regular water-based culture media; second, after treatment of the cells with H_2O_2, there was lower levels of apoptosis of the cells cultured in the KW-based culture media, compared to the cells in the regular water-based culture media; and the third, drinking of KW led to significantly decreased MPO activity in the mouse model of DSS-induced acute colitis, which is a key oxidative stress-generating enzyme in the inflammatory processes. Collectively, both our i*n vitro* studies and *in vivo* studies have indicated that the water produced by standardized Koisio technology has significant antioxidant capacity. Our study has also provided evaluations of the antioxidant capacity of the water produced by standardized Koisio technology.

Our previous study showed that the water that was produced by a preliminary Koisio technology showed certain levels of antioxidant capacity [8], which had proven the principle that Koisio technology-produced water has antioxidation capacity. However, since the preliminary Koisio technology was not a standardized procedure, it was not possible to produce water with stable quality using the technology, thus preventing us from obtaining quantitative evaluations of the antioxidant capacity of the water. Since currently the standardized procedures of Koisio technology for producing water has been established, we are capable of using the water produced by the technology to obtain quantitative evaluations of the antioxidant capacity of the water. Obviously, the findings from our current study represent a crucial progress on the biological properties of Koisio technology-produced water.

It is of significance to investigate the mechanisms underlying the antioxidant capacity of the Koisio technology-produced water. It is noteworthy that the MPO activity of the DSS-administered mice that had drunken the standardized Koisio technology-produced water was significantly lower, compared with the MPO activity of the DSS-administered mice that had drunken the regular water. It is of interest to determine if the standardized Koisio technology-produced water produced directly or indirectly the decreases in the MPO activity.

References:

[1] Á. Chamorro, U. Dirnagl, X. Urra, and A. M. Planas, "Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation," The Lancet Neurology, vol. 15, no. 8, pp. 869-881, 2016, doi: 10.1016/S1474-4422(16)00114-9.

[2] S. Cadenas, "ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection," Free Radical Biology and Medicine, vol. 117, pp. 76-89, 2018/03/01/ 2018, doi: https://doi.org/10.1016/j.freeradbiomed.2018.01.024.

[3] K. Luc, A. Schramm-Luc, T. J. Guzik, and T. P. Mikolajczyk, "OXIDATIVE STRESS AND INFLAMMATORY MARKERS IN PREDIABETES AND DIABETES," (in English), JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, vol. 70, no. 6, pp. 809-824, DEC 2019, doi: 10.26402/jpp.2019.6.01.

[4] T. Finkel and N. J. Holbrook, "Oxidants, oxidative stress and the biology of ageing," Nature, vol. 408, no. 6809, pp. 239-247, 2000/11/01 2000, doi: 10.1038/35041687.

[5] W. Ying and Z. G. Xiong, "Oxidative Stress and NAD⁺ in Ischemic Brain Injury: Current Advances and Future Perspectives," CURRENT MEDICINAL CHEMISTRY, vol. 17, no. 20, pp. 2152-2158, JUL 2010, doi: 10.2174/092986710791299911.

[6] X. Liu, Z. Pei, Z. Zhang, Y. Zhang, and Y. Chen, "Associations of Boiled Water

and Lifespan Water Sources With Mortality: A Cohort Study of 33,467 Older Adults," (in English), Frontiers in Public Health, Original Research vol. 10, 2022-June-27 2022, doi: 10.3389/fpubh.2022.921738.

[7] T. Feng, Z. Feng, Q. Liu, L. Jiang, Q. Yu, and K. Liu, "Drinking habits and water sources with the incidence of cognitive impairment in Chinese elderly population: The Chinese Longitudinal Healthy Longevity Survey," Journal of Affective Disorders, vol. 281, pp. 406-412, 2021/02/15/ 2021, doi: https://doi.org/10.1016/j.jad.2020.12.044.

[8] M. Zhang, Y. Men, Q. Zhu, and W. Ying, "Koisio Technology-Modulated Cell Culture Media can Significantly Increase the Antioxidant Capacity of Mouse Fibroblasts," bioRxiv, p. 2022.03.03.482792, 2022, doi: 10.1101/2022.03.03.482792.

[9] J. J. Kim, M. S. Shajib, M. M. Manocha, and W. I. Khan, "Investigating intestinal inflammation in DSS-induced model of IBD," (in eng), J Vis Exp, no. 60, Feb 1 2012, doi: 10.3791/3678.

[10] A. Khan et al., "Ameliorative effect of two structurally divergent hydrazide derivatives against DSS-induced colitis by targeting Nrf2 and NF-κB signaling in mice," (in eng), Naunyn Schmiedebergs Arch Pharmacol, vol. 395, no. 10, pp. 1167-1188, Oct 2022, doi: 10.1007/s00210-022-02272-w.

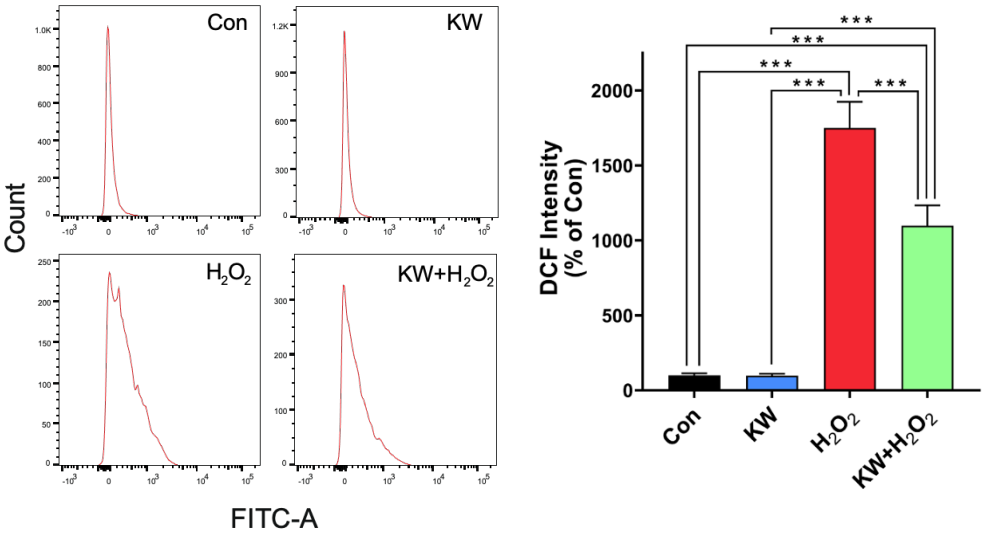
Figure Legends:

Fig. 1. KW showed significantly greater capacity of decreasing ROS levels in the H_2O_2-treated BV2 microglial cells. After BV2 cells were cultured in either regular cell culture media or KW-based cell culture media for 24 h, the cells were exposed to the 1 mM H_2O_2 prepared in MEM for 1 h. After the treatment, the cells were cultured in regular cell culture media or KW-based cell culture media for 18 – 24 h. Subsequently FACS-based assay using DCFH as ROS probe was conducted. The FACS-based assay showed that the intracellular ROS level in the cells cultured in KW-based cell culture media was significantly lower than that in the cells cultured in regular cell culture media. **, $P < 0.01$; ***, $P < 0.001$. N = 9. The data were collected from three independent experiments.

Fig. 2 KW showed significantly greater capacity to decrease apoptosis level in the H_2O_2-treated BV2 microglial cells. After BV2 cells were cultured in either regular cell culture media or KW-based cell culture media for 24 h, the cells were exposed to the 1 mM H_2O_2 prepared in MEM for 1 h. After the treatment, the cells were cultured in regular cell culture media or KW-based cell culture media for 18 – 24 h. Subsequently FACS-based assay was conducted with Annexin V Apoptosis Detection Kit with 7-AAD. The FACS-based assay showed that both early apoptotic cells and late-stage apoptotic cells cultured in KW-based cell culture media were significantly less than the cells cultured in regular cell culture media. **, $P < 0.01$; ***, $P < 0.001$. N = 9. The data were collected from three independent experiments.

Fig. 3 Drinking of KW significantly attenuated the increases in MPO activity in the mouse model of DSS-induced acute colitis. DSS induced significant increases in MPO activity, which was significantly attenuated in the mice that had drunk KW. ***, $P < 0.001$. N = 11 - 20.

Fig. 1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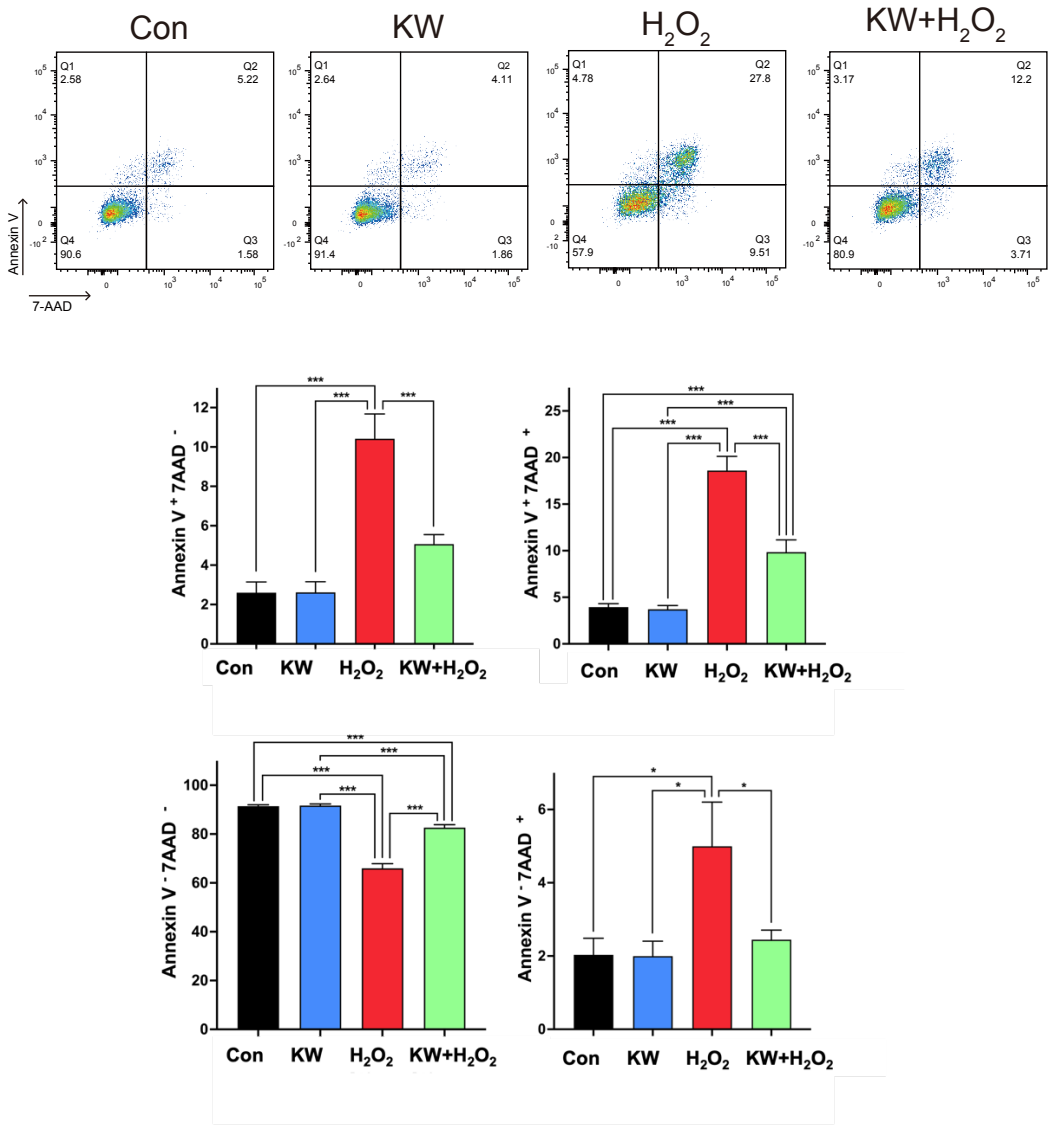
KW+H_2O_2

Fig 2.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Q2 27.8
Q4 57.9
Q3 9.51
Q1 3.17
Q2 12.2
Q4 80.9
Q3 3.71
Annexin V
7-AAD
Annexin V + 7AAD -
Annexin V + 7AAD +
Annexin V - 7AAD -
Annexin V - 7AAD +
Con
KW
H2O2
KW+H2O2

Fig 3.